Investigation of Neurovascular Inflammation Associated with Metabolic Syndrome and Depression in Rat Models of Alzheimer's Disease

Synopsis

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1. INTRODUCTION

The healthy survival of approximate 86 billion neurons in human brain depend on the structural and functional integrity of neurovascular system, which comprises blood vessels to provide nutrients, oxygen, astrocytes and microglia for maintenance, protection, and communication to the neurons.[1,2,3,4] The blood-brain barrier (BBB) formed by microvascular endothelial cells and sheathed by pericytes, and astrocyte end-feet is crucial for the protection of neurons from the factors present in systemic blood circulation.[1,5] BBB maintains the highly regulated composition of interstitial fluid, which is required for optimal synaptic connectivity, information processing, and neuronal functioning.[4] Breakdown of BBB integrity, as observed in multiple neurodegenerative diseases, enables the influx of neurotoxic blood-derived molecules, cells, and pathogens, which initiate inflammatory and immune responses.[2,3,6] Loss of BBB activates the perivascular macrophages and microglia, which surround the brain blood vessels.[7,8] In addition, infiltration of peripheral monocytes and macrophages as well as activation of resident microglia and astrocytes enhances the inflammatory milieu around the blood vessels.[9,10,11] This neurovascular inflammation is positively correlated with the neuronal loss and progression of several neurodegenerative diseases.[2,5,12] Alzheimer's disease (AD) is a chronic neurodegenerative disorder and the primary cause of dementia, which is rapidly emerging as one of the most burdening diseases of the century.[13] AD causes a gradual decline in memory, cognitive ability, behavior, personality, and ability to perform daily tasks.[14,15] The primary histological hallmarks of AD are amyloid-beta (Aβ) accumulation in brain parenchyma and around brain blood vessels, and neurofibrillary tangles formed by hyperphosphorylated microtubule-related protein tau in neurons.[16] Aβ aggregates and neurofibrillary tangles are neurotoxic and induce neuroinflammation, neurovascular dysfunction, and neurodegeneration.[13,15,17] Before the onset of clinical symptoms of dementia, the initial phase of disease progression involves neuroinflammation and vascular dysfunction, which is upstream or in parallel to accumulating Aβ aggregates and neurofibrillary tangles.[2,3,5,12,18] Blood-brain barrier disruption is linked with rapid cognitive decline in AD. Neurovascular impairment, especially blood-brain barrier dysfunction and cerebral blood flow shortfall is detected decades before the onset of symptoms.[4,17] Adequate supply of blood, and structural and functional integrity of blood vessels is a key to normal brain functioning. Changes in cerebral blood flow, leaks in the protective blood-brain barrier, and loss

of neurovascular integrity may lead to mild cognitive impairment (MCI), and AD.[4,17,19] Recent animal studies have suggested infiltration of monocytes across the blood-brain barrier into the brain and activation of microglia in the brain facilitates neuroinflammation.[11,20] Studies in the past decade have correlated neuroinflammation mediated with chronic glial activation with neuronal cell death and cognitive impairment in AD.[21] There is also a growing body of evidence that implicates systemic inflammation to the severity of neuroinflammation and AD.[11,20,22] Hyperactivation of systemic monocytes and macrophages contributes to cognitive impairment in Alzheimer's disease.[23]

Until recently, the most recognized explanation for AD pathology was the assumption that Aβ aggregation is an initial and critical pathogenic process in AD, contributing to the neurofibrillary tangles, neuroinflammation, synaptic degeneration, neuronal cell death, and dementia.[24] However, this model centered on Aβ is more applicable to the hereditary form of AD, found in younger people, as compared to the sporadic form of disease, which constitutes more than 95% of AD. Another explanation of AD pathogenesis suggests that initial neurovascular dysfunction precedes and impacts amyloid and tau alterations in the brain.[4] Studies at the molecular level have shown that brain vessels in AD express diminished levels of LDL receptor-related protein-1 (LRP-1), a key receptor responsible for transvascular Aβ clearance.[26] A diminished level of LRP1 receptor results in reduced Aβ transport out of the brain, leading to its intracerebral accumulation. In addition, a cerebral blood vessel in AD also expresses high levels of receptor for advanced glycation end products (RAGE), which transport Aβ in the opposite direction to LRP1 and moves systemic Aβ into the brain thus leading to enhanced intracerebral accumulation of toxic Aβ.[27] RAGE is also a major factor in the induction of oxidative stress and mediates the secretion of endothelin-1, which can cause vasoconstriction, diminished cerebral blood flow, and cognitive dysfunction.[28] In addition, the accumulation of advanced-glycation end products (AGE), a ligand for RAGE, in the brains of AD patients activates RAGE and promotes neurodegeneration.[29]

Aging is the primary cause of late-onset AD, which contributes to 95% of AD cases.[30] Recently, there has been consensus that stochastic factors (exposure to environmental and lifestyle factors) have a significant contribution to AD progression.[15,31] A study by the Lancet Commission on the prevention, intervention, and care, identified nine major modifiable risk factors for dementia; diabetes, obesity,

hypertension, depression, physical inactivity, low social contact, illiteracy, hearing impairment and smoking.[15] Among these risk factors, diabetes, obesity and hypertension are part of metabolic syndrome. The presence of any of the three risk factors, such as hyperglycemia, hypertension, hyperlipidemia, hypercholesterolemia, and obesity, is defined as metabolic syndrome.[32] Long-term consumption of diets with high fat and sugar can lead to metabolic syndrome, and several studies with human and experimental rodents have strongly implicated such diets with metabolic syndrome and AD.[33,34,35] Studies with human and experimental rodents have established that Western diets or modern cafeteria diets, which contain high-fat-sugar-salt (HFSS) can increase the metabolic risk factors, such as hyperglycemia, hypertension, hyperlipidemia, hypercholesterolemia.[36,37] Metabolic syndromes are often associated with type 2 diabetes, which has a bidirectional relationship with AD. Type 2 diabetes can cause impairment of vascular function and neurodegeneration.[38,39] Nearly 80% of people with Alzheimer's dementia have some form of diabetes. The elderly with metabolic syndrome often share some of the neuropathological features with AD, for instance, impaired insulin signaling, enhanced oxidative stress, Aβ plaques, tau, and neurovascular dysfunction.[38] High blood sugar levels in metabolic syndrome enhance the glycosylation process thus increasing the formation of AGEs, which enhances the activation of RAGE.[40] In patients with AD and metabolic syndrome comorbidity, there is much more pronounced neuroinflammation, neurovascular impairment, and neuronal dysfunction. However, the precise status of neurovascular inflammation in AD and metabolic syndrome comorbidity is not well understood.

Depression is another modifiable risk factor commonly associated with AD.[41] Depression in AD is a heterogeneous disorder with complex etiology and symptoms.[42] Depressive symptoms affect around half of the Alzheimer's patients during the disease.[43] The presence of comorbidity of depression increases AD pathology and causes cognitive decline.[44] Comorbid depression in AD causes greater impairment in daily living activities thus adversely affecting the quality of life of patients. Patients with neuropathologically confirmed AD with comorbid depression show brain changes with higher levels of neuritic plaques and advanced-stage cortical neurofibril tangles.[45] The brains from both AD and depression are associated with enhanced inflammation, which induces the IL1β - the master regulator of neuroinflammation.[46,47] Inflammation seems to be a common link between AD and depression;

however, it has not been explored in the context of localized neurovascular inflammation in Alzheimer's disease comorbidity with depression.

Medicine may improve or slow the progression of symptoms; however, there is no treatment that cures AD.[13] With the dramatic change in the lifestyle of Indians, there is an increasingly high prevalence of metabolic syndrome and depression, which could adversely affect the onset and progression of AD.[48] Both depression and metabolic syndrome are associated with the induction of neurovascular dysfunction and neuroinflammation, which contribute to neurodegeneration in AD. Despite extensive research on various causative and contributory factors for non-familial AD, the impact of AD comorbidities with metabolic syndrome and depression on neurovascular inflammation is not well known. We aim to investigate the neurovascular inflammation in comorbidities of metabolic syndrome and depression in rat models of AD.

2. REVIEW OF LITERATURE

2.1 Neurovascular system: Neurons are central to cognitive ability, however, for the adequate functioning of neurons in the human brain a 400 miles long network of blood vessels provides oxygen and nutrients, and facilitates exchanges of metabolites, hormones, and other essential molecules.[5] The cerebral venous return extracts carbon dioxide and metabolites out of the brain.

***Neurovascular coupling*:** The brain is considered an energy-hungry organ and to fulfill its demand brain requires the constant supply of nutrients for proper neuronal functioning.[49] When the localized metabolic demand of neurons for nutrients increases during brain activity, it induces a further increase in cerebral blood flow (CBF) in that brain region, which is known as neurovascular coupling. Impairment of neurovascular coupling of several neurodegenerative diseases, such as stroke and Alzheimer's dementia can cause neuronal damage.[17]

Neurovascular unit: The neurovascular unit consists of endothelial cells, mural cells (pericytes and smooth muscle cells), glia (astrocytes and microglia), and neurons. The blood vessels work as a part of the neurovascular unit, which comprises endothelial cells, which form inner layer of blood vessels, mural cells attached to the vessels to regulate the blood flow (pericytes and vascular smooth muscle cells), astrocytes to enable communication between blood vessels and neurons, and microglia for immune function.[5] Neurovascular unit precisely regulate cerebral blood flow to ensure that localized and

specific brain energy demands are met.[17] The neurovascular unit has a unique communication between neurons and the vascular system through astrocytes to enable vasodilation to facilitate the localized demand for nutrients.[1] Astrocytes across mammals, from rodents to humans, the neurovascular unit is polarized and astrocyte end-feet firmly attached to the endothelial cells at blood vessels to enhance the integrity of BBB.[50] In addition, the mammalian brain also shows remarkable anatomical interaction of astrocytes end feet with pericytes on blood vessels, which through calcium signaling enable the neuronally evoked capillary dilation.[51]

Neurovascular inflammation: Neuroinflammation, where infiltration of peripheral immune cells and activation of brain microglia and astrocytes promotes the dysfunction of the neurovascular unit is called neurovascular inflammation. The neurovascular inflammation impairs neurovascular coupling and enhances neuronal loss, which can lead to cognitive deficits in several neurodegenerative diseases, such as stroke and Alzheimer's dementia.[3,5]

2.2 Alzheimer's dementia*:* Alzheimer's disease is the most common form of dementia. It is a progressive neurodegenerative disease with a gradual decline in memory, thinking, behavior, and social skills.[5] Alzheimer's disease involves parts of the brain that control thought, memory, and language. In advanced stages of AD, severe degeneration of brain functions makes it difficult for the patient to move, sit up, and even swallow, resulting in dehydration, malnutrition, impaired immunity, and infection such as aspiration pneumonia, a leading cause of death.15 Dementia leads to premature death and the risk of mortality increases to double than without dementia.52,53 Worldwide more than 50 million people live with dementia, and this number is expected to increase to 150 million by 2050, rising particularly in low- and middle-income countries, such as India and China.[15] India has the world's highest population, and with an increasing lifespan and decreasing fertility rate, by 2050, about one-fifth of Indian population will be over 60 years of age.

Pathogenic markers of Alzheimer's disease: AD is characterized by Aβ accumulation in brain parenchyma and around brain blood vessels, and neurofibrillary tangles formed by hyperphosphorylated microtubule-related protein tau in neurons.[16] Aβ and neurofibrillary tangles are neurotoxic and induce neurodegeneration.[15]

Amyloid beta and tau. Amyloid peptides aggregate into oligomers, fibrils, and bigger aggregates in the

form of plaques. Amyloid oligomers are considered the most toxic species of Aβ. Macrophages and microglia are primary scavengers in the brain and degrade the Aβ aggregates through lysosomal pathways. Aβ accumulates in the brain decades before the first symptoms in a stepwise manner, starting first in the temporobasal and frontomedial lobes, and then spreading to the neocortex, the primary sensory-motor cortex, and then the striatum.[54]

The clearance of toxic Aβ peptides relies on transport to the cerebrospinal fluid and the blood through BBB, both of which are impaired in AD. LRP-1 transport Aβ from the brain to the blood, where it is cleared out through the liver and kidneys.[4] RAGE transports the Aβ from the blood into the brain, resulting in the influx of peripheral Aβ into the brain.[4,17] Neuronal death in AD is associated with aggregation and polymerization of tau protein into intracellular paired helical filaments, which form larger neurofibrillary tangles.[55] Recently, tau has been implicated in BBB leakage in AD.[56]

Aβ-induced pathogenic mechanisms: The accumulation of toxic amyloid peptides, primarily Aβ40 and Aβ42, is the initial and critical event in AD, initiating neuroinflammation, vascular dysfunction, and neuronal loss, which leads to cognitive impairment (Figure 1, illustrated by Soni Tiwari and inspired from reference number 54). Only less than 5% of AD causes are genetic and caused due to genetic mutations, such as amyloid precursor protein on chromosome 21, presenilin 1 on chromosome 14, and presenilin 2 on chromosome 1.[54] Familial cases of AD result in early-onset amyloid accumulation in the brain and symptoms appear as early as in the thirties. However, most AD cases are late-onset and symptoms appear later than 60 years of age, even though the accumulation of Aβ in the brain starts more than two decades earlier. The precise trigger for Aβ accumulation is unclear, however, aging, ApoE genotype, inflammatory phenotype, and unhealthy lifestyle are considered major risk factors.[54] Evidence from recent studies has questioned the amyloid cascade hypothesis. There are reports that not all people with amyloid deposition develop AD, including those with Down's Syndrome.[16,54] Cognitive impairment is often associated with atrophy in the entorhinal cortex and is present before the amyloid deposition. More than 30% of cognitively normal people over the age of 70 years and more than 50% of people over 95 years of age have elevated amyloid in CSF.[54] A unifying acceptable mechanistic model that includes the role of Aβ and other risk factors is still missing.

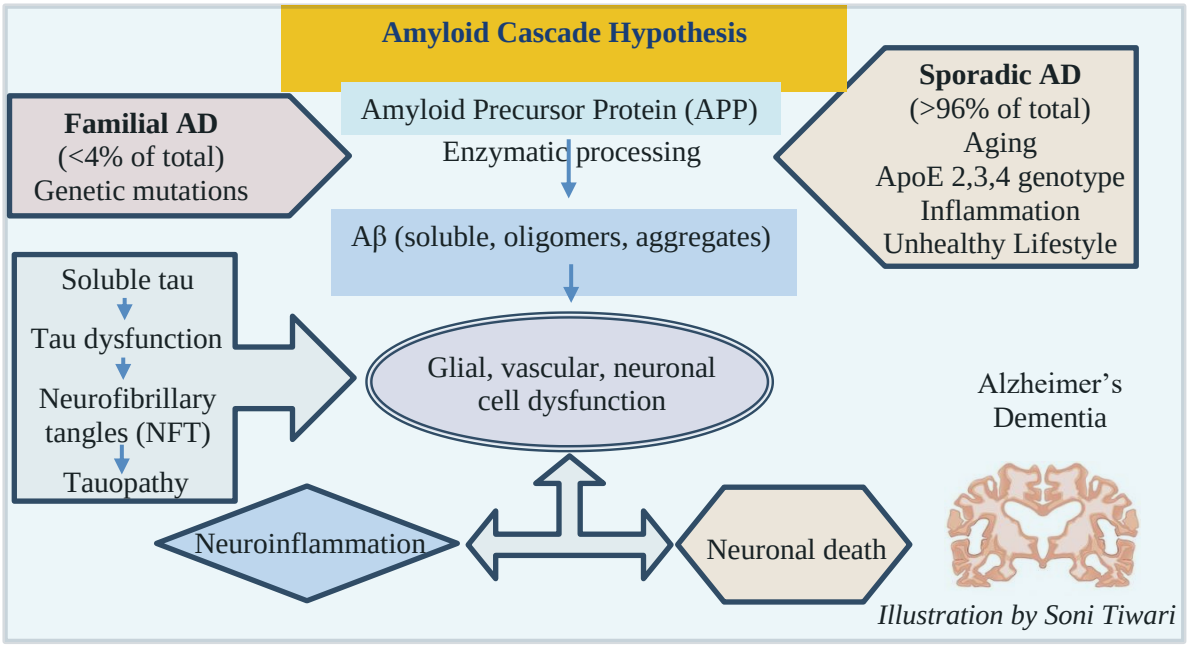

Figure 1. Amyloid cascade hypothesis postulates that the neurodegeneration in Alzheimer's disease is caused by abnormal accumulation of Aβ into the brain. Further, this hypothesis suggests that Aβ accumulation is central to the neuroinflammation, neurovascular dysfunction and neuronal cell death.

2.3 Dysfunction of the neurovascular unit in Alzheimer's disease: There is strong evidence that neurovascular dysfunction, Cerebral blood flow reductions, and dysregulation and breakdown of the BBB, including the loss of pericytes, are early events in the AD pathophysiological cascade.[4] The two-hit vascular hypothesis proposed that vascular dysfunction precedes the amyloid and tau alterations in the brain (Figure 2, illustrated by Soni Tiwari).[4] Aβ oligomers induce vasoconstriction thus reducing CBF and likely play a critical role in the dysfunction of neurovascular unit in AD. Improperly functioning blood vessels fail to deliver essential nutrients, including oxygen and glucose, and remove metabolic waste products from the brain.[17] In the event of halting of CBF, functions cease within seconds and permanent brain damage occurs in minutes.[17]

2.4 Contribution of BBB leakage to neurovascular inflammation: The BBB restricts entry of blood-derived products and pathogens from entering the brain and regulates molecule entry into and out of the brain. Studies with brain imaging found increased BBB breakdown in individuals with early-AD and identified perivascular microbleeds in mild cognitive impairment and early AD.[57] Post-mortem human studies demonstrated reduced capillary length and microvascular degeneration with diminished tight junction proteins.[17] Additionally, microvascular leakages of blood-derived products including fibrinogen, immunoglobulin G (IgG), thrombin, albumin and hemosiderin increase neurovascular

inflammation of AD.[57] Deposition of amyloid peptide in blood vessels in AD brain is also associated with decrease in pericyte coverage and breakdown of tight junction proteins. Systemic inflammation often increases BBB permeability in AD, thus allowing immune mediators, like monocytes and macrophages to enter the brain.[57] Taken together, there is clear evidence that AD is associated with neurovascular inflammation. However, the impact of comorbidities such as metabolic syndrome and depression on neurovascular inflammation in AD is not well understood.

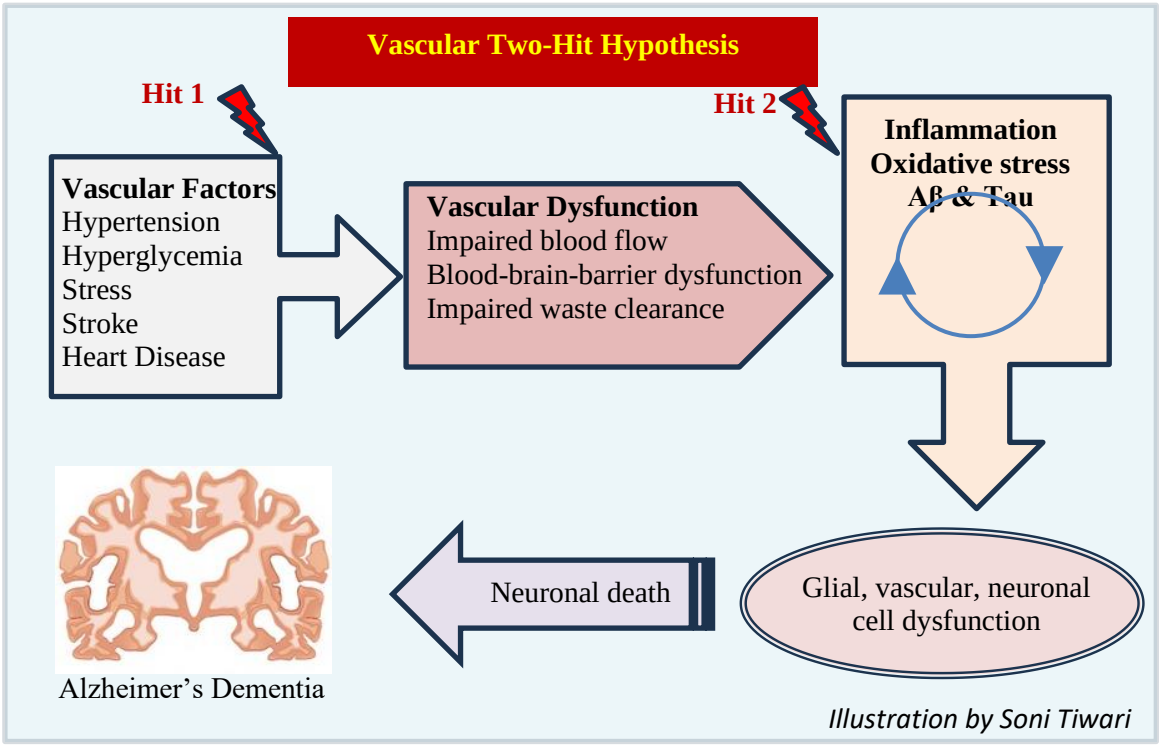

Figure 2. Vascular two-hit hypothesis postulates that the neurovascular impairment leads to neuroinflammation, oxidative stress, which promotes the abnormal accumulation of Aβ and tau tangles into the brain. Further, this hypothesis suggests that vascular dysfunction directly and through induction of Aβ accumulation leads to neuronal cell death and dementia in Alzheimer's disease.

2.5 Diet-associated cardiovascular risk factors in neurodegeneration: Modern diet is often associated with intake of unhealthy diets with high-fat-sugar, which is a major risk factor for metabolic syndrome and diabetes, which aggravates the AD pathology.[22] Among the modern diets, "junk food" is defined as a hyperpalatable food rich in calories and containing high sugar, fat and salt, however, with low fibers, protein, vitamins and minerals.[59] Another example of modern diet is "Western diet", which is prevalent in western countries and defined as ultraprocessed food containing high amounts of refined sugars, saturated fats, salt and protein from animal sources.[59] Junk food and Western diets are often defined as high-fat-sugar-salt diet or HFSS diet. Diet with high amount of sugar and fat is associated with reduced

levels of brain proteins involved in synaptic plasticity, such as acetylcholine, dopamine, serotonin, and brain-derived neurotrophic factor.[22,60] Further, high fat and sugar diet is associated with decreased overall volume of the left hippocampus and cognitive impairment.[22,60,61] These conditions induce a peripheral low-grade inflammatory state, characterized by high levels of peripheral pro- inflammatory cytokines.[22] Western diets or diets with high-fat-sugar-salt can induce diabetes, hypercholesterolemia, obesity, non-alcoholic fatty liver disease, hyperglycemia, insulin resistance and high blood pressure and other vascular disorders, which are known risk factors for metabolic syndrome as well as AD.[22] A diet with reduced AGE can benefit older people with mild cognitive impairment and metabolic syndrome comorbidity.[62]

2.6 Metabolic syndrome-associated neurodegeneration in AD: Recent studies have shown that metabolic syndrome comorbidity enhances AD neuropathological features, and impairs cognition, learning, and memory.[63,64] South Asians, including Indians, are predisposed to metabolic syndrome even at lower weight and younger ages than in Western countries.[65] Hyperglycemia induces the formation of AGEs and its receptor RAGE, which are considered a major risk factor for neurodegeneration AD.[66] However, the mechanism underlying the neurovascular inflammation in AD and diabetes comorbidity is not well understood. Metabolic syndrome-associated conditions, such as hyperglycemia, hyperlipidemia, and hypertension cause endothelial and vascular smooth cell dysfunction, which impairs neurovascular coupling and leads to cognitive impairment.[22,63,64]

AGE activates RAGE receptor, which is also induced by Aβ and causes oxidative stress and inflammatory reactions.[67] RAGE facilitates intracerebral amyloid accumulation by transporting Aβ across the blood vessels into the brain parenchyma.[27] Cerebral vessels of AD patients have elevated levels of p65 RelA linked with RAGE-cascade.[68] Metabolic syndrome is also associated with activation of p65 RelA which plays an important role in inflammatory cascade.[69] Persistent hyperglycemia is the primary underlying factor responsible for the oxidative stress and neuroinflammation. The higher score of metabolic syndrome is strongly associated with oxidative stress, systemic inflammation and neuroinflammation, all of which contribute to early cognitive impairment.[70]

Rodent models of AD comorbidity with metabolic syndrome: Rodent serves as an excellent model for metabolic syndrome and vascular dysfunction. Comparative proteomic analysis has shown that rodent

brains share similarities with human brains in terms of various neurovascular proteins.[71] However, in nature, rodents do not develop aggressive amyloid aggregates or Alzheimer's pathology like humans. So, a greater understanding of strengths and weakness of rodent models should be taken into consideration. The inclusion of cell culture models can confirm or supplement the research outcome. To induce amyloid aggregation in rodents, either human mutations or amyloid precursor protein needs to be inserted in the genome of rodents or amyloid peptide itself needs to be injected into the brain.[72] Providing rats with a diet with more than 45% fat can induce metabolic syndrome, however, a diet with high-fat-salt-sugar (HFSS) can show the higher grade of metabolic syndrome, with hyperglycemia, hyperlipidemia, hypertension, and insulin resistance.[73,74]

2.7 Depression-associated neurodegeneration in AD: Major depressive disorder is a phenotypically complex heterogeneous disorder that genetically overlaps with Alzheimer's with the influence of environmental factors.[75] Depression is the most frequent neuropsychiatric symptom in mild cognitive impairment and the most frequent neuropsychiatric symptom across different stages of AD.[76] It is known that major depressive disorder plays a key role in the early as well as late onset of variants in dementia, and there is not only a psychological connection alone but a neurobiological connection as well which increases the prevalence of late onset of AD.[75,77] Among all the psychological disorders in India, depression is the most prevalent and causes about 34% of all cases of mental disorders (Sagar et. al. 2020). The contribution of depressive disorders to the total disability-adjusted life years is substantially higher in females than in males.[78] In 2017, the World Health Organization reported that depression was the largest contributing factor to global disability affecting around 322 million[79] and the main cause of suicide deaths, which is often ignored.[80] Among nine modifiable serious risk factors, depression predisposes to the aggravated pathology of AD.[15] It is well established that major depressive disorder increases the risk of AD through the activation of proinflammatory cytokines.[81] Neuropathological studies of the postmortem hippocampus from patients with a lifetime history of depression with AD showed that the neuritic plaques and neurofibril tangles are more pronounced in AD comorbidity with depression.[82] Neuroinflammation is more pronounced in both Alzheimer's and depression.[83,84] It is well known that both innate and adaptive immune systems are dysregulated in depression, which leads to neuronal and vascular damage in the brain.[85] Microglial activation and hyperactivation of

proinflammatory milieu contribute to depression and cognitive deficits.[86] Human and animal studies show that various cytokines (IL1β, IL6, and IL8) and chemokines mediators play a key role in central and peripheral inflammation leading to the onset of depressive symptoms and cognitive dysfunction.[87] However, the impact of depression on neurovascular inflammation in AD has not been explored well.

Rodent models of AD comorbidity with depression: Animal models of depression range from non-human primates to zebra-fish; however, rodents remain the gold standard for the experimental studies for depression.[88] Depression is a multifactorial disorder with a wide range of heterogeneity. It is manifested more severely in females than males. The utility of the human depression scale DSM-5 for rodents can be problematic especially when assessing the symptoms like suicidal ideation.[88] Rodents show considerable differences within and between inbred and outbred strains. Consequently, all these factors should be taken into consideration to test a hypothesis on depression in rodents. Chronic social defeat, early life stress, loss of enrichment, and chronic unpredictable stress paradigms are often applied to induce depression in rodents.[88]

An emerging role of brain vasculature in the pathogenesis of human neurodegenerative diseases, particularly AD, has led to the increasingly recognized importance of healthy blood vessels for normal brain functioning. Metabolic syndrome and depression can be a major modifiable risk factor for AD. A better understanding of neurovascular inflammation in metabolic syndrome and depression can help in identifying potential molecular targets, which could lay the foundation for developing better diagnostics and therapeutics for AD.

3. OBJECTIVES

Modifiable risk factors, especially metabolic syndrome and depression are considered as major risk factors for AD.[15,89] The long-term consumption of HFSS/Weste rn diet can lead to hyperglycemia, hyperlipidemia, hypertension, and insulin resistance, which are major vascular risk factors for AD. HFSS/Western diets are associated with an increase in harmful AGEs, which act on RAGE receptors to induce neurovascular impairment and cognitive dysfunction.[67] Neuroinflammation is a common feature in metabolic syndrome, and depression comorbidities with AD.[46,90] Neuroinflammation is known to advance neurovascular degeneration in AD. *Our primary hypothesis is that metabolic syndrome and depression could potentiate neurovascular inflammation and cognitive impairment in AD.* We would test

our hypotheses under the following specific objectives:

Objective 1. To investigate the impact of metabolic syndrome on neurovascular inflammation and cognitive dysfunction in a rat model of AD.

Objective 2. To identify the potential molecular events underlying neurovascular inflammation in AD comorbidity with metabolic syndrome using cell culture and rat models.

Objective 3. To assess the impact of depression on neurovascular inflammation and cognitive dysfunction in a rat model of AD.

Objective 4. To determine the molecular events underlying neurovascular inflammation in cell culture and rat model of AD comorbidity with depression.

4. MATERIAL AND METHODS

4.1 Material

***Cells*:** Human brain microvascular endothelial cells, human brain smooth muscle cells, THP-1 monocyte cell line, HMC3 microglia cell line from ATCC.

***Animals*:** Sprague-Dawley rats from the National Institute of Biologicals, Noida, UP, and All India Institute of Medical Sciences, New Delhi. We will conduct the rodent experiments at the animal facility of ACBR, Delhi university. Animal ethical approval has been submitted as Annexure 1. We have obtained required ethical approval 04/IAEC/ACBR/IS/March2023, and in each cage, upto 4 rats will be group-housed in a controlled environment (22 ℃–25 ℃, 50% humidity and 12 hours of light: dark cycle) with free access to diet and water.

***Media and Reagents*:** Endogrow media, DMEM media, RPMI media, RIPA lysis buffer, protease and phosphate inhibitors, dihydroethidium, acetic acid, thiobarbituric acid, potassium chloride, n-butanol: pyridine, Griess reagent, sodium nitrate and Aβ peptides (Aβ1-40 and Aβ1-42. The reagents would be of analytical grade.

Antibodies: The rat antibodies against advanced glycation end products, receptor for advanced glycation end products, metalloprotease 9, zona occludin 1 and glial fibrillary acidic protein and ionized calcium binding adapter molecule 1.

4.2 Methods

The methodology of above-mentioned objectives is as follows:

Objective 1. To investigate the impact of metabolic syndrome on neurovascular inflammation and cognitive dysfunction in rat model of AD.

Using Sprague-Dawley (SD) rats, we will develop an animal model of AD comorbidity with metabolic syndrome. We will perform following steps:

***Step 1*:** *Generation of AD-metabolic syndrome comorbidity model:* First, at 2 months of age, we will inject Aβ (25 μg) intracerebroventricular into the rat brain by stereotactic apparatus. Thereafter, rats will feed a high fat (45%)-sugar (30%) diet for 8 months to develop AD-metabolic syndrome comorbidity. We will have 4 groups of animals: control, metabolic syndrome, AD, and AD comorbidity with metabolic syndrome (n=8 per group, total 32 animals).

Step 2: *Characterization of model:* To determine metabolic syndrome in the SD rats after feeding high-fat-sugar diet for 8 months, we will assess the blood glucose, blood triglyceride, blood HDL, glucose tolerance and HbA1c from the tail vein blood. Thereafter, we will conduct the neurocognitive analysis on all the rats. At the end animals will be sacrificed to determine the AD pathology by histological analysis of brain sections.

***Neurocognitive tests*:** To determine the neurocognitive effect of metabolic syndrome (high-fat-sugar diet) on control and AD animals, we will perform the behavioral tests, such as Y-maze, novel-object location, novel object recognition tests for recognition memory between novel and familiar. The nesting and burrowing tests will determine the rodent's innate behavior. The methods, in brief, are as follows:

***Novel object recognition test*:** A novel object location test (NOL) assesses spatial hippocampus-dependent memory, and a novel object recognition test (NOR) assesses perirhinal cortex-dependent memory.[91] For the NOR test, SD rats will be allowed to explore the arena for 5 minutes with two identical sample objects with the same color and texture. Rats will be taken out, and one of two identical objects was replaced with a novel object with a different color and texture. After a 10-minute inter-trial period, rats will be returned to the arena, and object exploration behavior will be assessed for 5 min. Then, the percent exploratory preference and discrimination index will be calculated.[91]

Novel object location test: For the novel object location or NOL test, SD rats will be placed into the arena with two identical objects and allowed to explore for 5 min. Rats will then be taken out and one of the objects will be placed in a new location. After a 10 min inter-trial period, rats will be returned to

the arena and novel location exploration behavior will be measured for 5 min. Then, the percent of exploratory preference and time spent with newly placed object will be calculated.

Y-Maze: This test can be used to assess spatial working as well as reference memory in rodents. Rats will be placed into one of the arms of the maze (start arm) and allowed to explore two of the arms for 5 min (training trial).[91] The third arm, which remained closed, will be randomly chosen in each trial. After a 30-minute inter-trial interval, the blocked arm will be opened, and rats will be put back to the start arm and allowed to explore all three arms for 10 min (test trial). The number of novel arm entries will be calculated.

Burrowing task: SD rats will also be tested for hippocampal-dependent burrowing behavior analysis. At the onset of the dark cycle SD rats will be individually placed in cages equipped with a burrow made of a 250 mm long and 80 mm diameter plastic tube.[91] The burrow will be filled with 300 g of rat food pellets, and rats will be allowed to burrow for 3 hours. The weight of the remaining food pellets inside the burrow will be determined to obtain a measurement of the amount burrowed.

Nesting: SD rats will also be tested for hippocampal-dependent nesting behaviour. The nesting experiment will be performed by placing the rat with 3 pieces of paper (5cmx5cm) in a cage with corn bedding.[92] Scoring will be as follows: 0 points for intact paper; 1 point for paper scattered throughout the cage, but no obvious bite marks (indicating active nest building); 2 points for paper concentrated in the cage, but no obvious bite marks; 3 points for the paper shredded but no nest building; 4 points for most of the paper shredded and gathered; and 5 points for paper shredded to a perfect nest with the crater in middle.[92]

Immunohistochemistry: Paraformaldehyde fixed brain tissue from SD rats will be transferred to 30% sucrose for 48 hours and sectioned to 40 μm thickness. The brain sections will be blocked for 2 hours in goat serum with tris-buffered saline and 0.1% Tween (TBST) and then incubated in primary antibodies (anti-GFAP, anti-Iba1, and anti-CD31). Washes will be performed, and the brain sections will be incubated with fluorescent labelled respective secondary antibodies. After washing, the brain section will be mounted using anti-fade fluorescence mounting media and imaged using a confocal microscope. The images will be analyzed using Image-J software.[91]

Objective 2. To identify the potential molecular events underlying neurovascular inflammation in AD

comorbidity with metabolic syndrome using cell culture and rat models.

To identify the molecular events, we will perform cell culture-based assays and Western blot. In addition, we will use the rat brains from the aforementioned rat models to identify the expression of various proteins by Western blot. The specific details are as follows:

Step 1. Maintenance of cells: We will purchase human brain microvascular endothelial cells and human brain smooth muscle cells from ScienCell Research Laboratories. Human brain vascular smooth muscle cells and human brain microvascular endothelial cells will be cultured in smooth muscle cells and endothelial cells complete medium. Cells from passages 4-6 will be used for the studies and will be maintained at 37°C at 5% CO_2.

Step 2**:** Both smooth muscle and endothelial cells will be incubated with 1μM Aβ40 and Aβ42.

Step 3**:** To determine the vascular dysfunction the cells will be subjected to the following assays:

Cytotoxicity Assays: First, we will perform a WST8 assay to assess the Aβ-mediated cytotoxicity. In this assay, the amount of the water-soluble formazan dye (orange colour) generated is directly proportional to the number of live cells.[93] An aliquot of 10 μl of WST8 reagent (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium) will be added into each well and absorbance at 450 nm would be taken to determine the live cells.

Thiobarbituric acid reactive substances (TBARS): Thiobarbituric acid reactive substances are formed during lipid peroxidation, and these are detected by the TBARS assay using thiobarbituric acid. A solution containing thiobarbituric acid will be added to each cell lysate and the mixture will be heated at 100°C for 60 minutes. TBARS will be extracted with 2.5 ml n-butanol: pyridine (15:1) and centrifuged to separate the organic and aqueous phases. The organic phase will be analyzed at 532 nm using a spectrophotometer to determine the lipid peroxidation.

Oxidative stress**:** The cells will be cultured in 96 well plates and oxidative stress will be assayed using the dihydro-ethidium (DHE). Oxidation of DHE by superoxide anions in cells hydrolyzes DHE and fluorescent ethidium is released, which bounds to the DNA in the cells.[94] Cells will be incubated with 5 μM DHE for 30 min at 37°C. Cells will be subsequently washed with phosphate buffered saline (PBS), lysed, and measured for fluorescence intensity at 570 nm.

Detection of nitric oxide bio availability by Griess reagent: Nitric oxide (NO) production will be

assessed based on the accumulation of its oxidative derivates, i.e. nitrates and nitrites in the cell culture medium using a colorimetric reaction with Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 0.1% sulphadiazine, and 2.5% H3PO4,[95] Initially, 50 µl of medium from each culture well will be placed in triplicate wells of a 96-well plate and then 100 µl of Griess reagent will be added to each well. After 10 minutes the optical densities will be measured at 540 nm.

Transendothelial electric resistance (TEER): The human brain microvascular endothelial cells would be used to prepare an *in vitro* model of BBB. Cells will be seeded in the transwell and allowed to grow for 10 days to enable full confluency in Endogrow media.[67,96] TEER will be measured periodically using EVOM2 chopstick electrodes (Millipore, Burlington, MA, USA) to monitor cell confluence and the development of tight junctions. Background resistances (R-BLANK) will be subtracted from the total calculated resistance R-TOTAL at each time point and normalized for the area, providing TEER values in Ohms (Ω.)/cm2 as in the following equation: R-TISSUE (Ω) = R-TOTAL − RBLANK; TEERREPORTED = R-TISSUE (Ω) × M-AREA (cm^2).

Western blot: Samples from cell homogenates from *in vitro* experiments will be lysed with sonication in cold radioimmuno precipitation assay (RIPA) lysis buffer supplemented with protease and phosphatase inhibitors cocktail.[97] The samples will be centrifuged at 20,000×g for 25 min at 4°C and the supernatant will be collected. The proteins from the supernatants will be separated on SDS–polyacrylamide gels (12%) in Tris/Glycine/SDS buffer and then transferred onto nitrocellulose membranes.[68] We would use antibodies against the following proteins for Western blot: advanced glycation end products (AGEs), receptor for AGEs (RAGE), intracellular adhesion molecule 1 (ICAM1), IL1β and zona occludin 1 (ZO1).

Step 4: *Analysis of rodent brain samples by a Western blot:* Samples from *in vivo* experiment (homogenized cortex and hippocampus of rat) will be subjected to analysis for AGE, RAGE, MMP9, ZO1, and ICAM1 by following the protocol mentioned above.

Objective 3. To assess the impact of depression on neurovascular inflammation and cognitive dysfunction in a rat model of AD.

We will develop an animal model of AD comorbidity with depression using SD rats. Animal will be group-housed in a controlled environment (22 °C–25 °C, 50% humidity and 12 hours of light: dark

cycle) with free access to diet and water. We will perform following steps:

Step 1**:** *Generation of AD-depression comorbidity model:* First, at 2 months of age, we will inject Aβ (25 μg) intracerebroventricular into the rat brain by stereotactic apparatus. Thereafter, rats will be subjected to unpredictable chronic mild stress for two months. We will have four groups of animals for the study: control, AD with amyloid injection, depression, AD-depression comorbidity rats with both AD and depression.

Step 2: *Characterization of model:* To determine depression in the SD rats after unpredictable chronic mild stress, we will perform light-dark tests, novelty suppressed feeding tests and forced swimming tests. At the end animals will be sacrificed to determine the AD pathology by histological analysis of brain sections.

Light-dark test: Light-Dark test will assess the animal's preference of choosing a light or dark area for rest.[47] This exploration test comprises two chambers, one with light and another dark-colored without light. Both chambers will be connected through the one center open door. The animal will be placed in the light chamber and left in the arena for 10 minutes. The percentage of time spent in the light area will be calculated to estimate the preference.[47]

Novelty-suppressed feeding test: Hyponeophagia (novelty-suppressed feeding) is widely used in the case of chronic and unpredictable chronic mild stress model of depression.[98] A food treat (highly palatable food) will be placed on a central platform in the middle of the apparatus. Experimental rats will be placed into the corner of the cage, and the latency for rats grasping the food treat with their forepaws and biting will be recorded with a timer for 10 minutes. The neglect of the food treat would assess the depression in the rats.

Forced swimming test: Force swimming apparatus is a clear glass long cylindrical 50 cm in height and 20 cm in diameter that can easily hold rodents.[99] On day one, the rat will be allowed to swim for 15 min and the next day (test day) the rat will be allowed to swim for 5 min. The time of immobility will be counted through the timer.

Neurocognitive tests**:** We will perform novel object recognition (NOR), novel object location (NOL), Y-maze tests, burrowing, and nesting as mentioned above.

Immuno-histochemistry: The brain sectioned from these *in vitro* experiments will be subjected to

analysis for staining with Iba1, GFAP, CD31-IgG, and ZO1 by following the protocol mentioned above.

Objective 4. To determine the molecular events underlying neurovascular inflammation in cell culture and rat model of AD comorbidity with depression.

***Step 1*:** We will grow the THP-1 monocyte cell line and HMC3 microglia cell line in RPMI 1640 media and maintain the cells at 37°C at 5% CO_2.

Step 2: The cells will be incubated with 1 μM Aβ40 and Aβ42

Step 3: Characterization of induction of inflammation will be carried out using the above mentioned WST8 cytotoxicity assay, oxidative stress dihydroethidum (DHE) assay, lipid peroxidation by TBARS.

Cytokine release ELISA assay: IL1β in the cell culture and rat's brain samples will be determined using commercially available ELISA kits. Anti-IL1β antibodies will be coated on 96 wells plate and samples will be incubated for 2 hours. The substrate working solution will be used for the colour generation, followed by the addition of a termination solution. Their corresponding concentrations will be calculated based on the specific OD value.[91]

Transmigration of monocyte in blood-brain barrier model: The endothelial cells will be grown on Transwell for two weeks to confluency. After the formation of a confluent monolayer in two weeks the THP1 patient-derived monocytes (20,000 cells) would be added to the upper chamber of ThinCerts. After 4 hours of incubation, the plates will be gently swirled and the media from the lower chamber will be sampled to determine the number of monocytes migrated to the lower chamber.

***Western blot*:** Samples from these *in vitro* experiments will be subjected to analysis for RAGE, and MMP9 by following the protocol mentioned above.

Step 4: *Analysis of rodent brain samples by Western blot:* Samples from *in vivo* experiment (homogenized cortex and hippocampus of rat) will be subjected to analysis for AGE, RAGE, MMP9, ZO1, and ICAM1 by following the protocol mentioned above.

***Statistical analysis*:** The statistical analysis will be performed using PRISM statistical software package. One-way analyses of variance (ANOVA) will be used to find statistically significant differences among experimental groups. Bonferroni test will be used for post-hoc ANOVA analysis. Student's t-test will be used for statistical comparison between the two groups. A p value < 0.05 will be considered statistically significant. To establish the relationship between depression with neurovascular inflammation, and

metabolic risk factors with neurovascular inflammation, we would perform multiple regression and Pearson correlation analysis.

5. ABBREVIATIONS

Alzheimer's disease: AD

Blood-brain barrier: BBB

Amyloid-beta: Aβ

Mild cognitive impairment: MCI

LDL receptor-related protein 1: LRP-1

Advanced-glycation end products: AGE

Receptor for advanced glycation end products: RAGE

High-fat-sugar-salt: HFSS

Interleukin 1 beta: IL1β

Beta-site amyloid precursor protein-cleaving enzyme 1: BACE-1

Soluble extracellular soluble fragment of APP: sβAPP

Cerebral blood flow: CBF

Neurofibrillary tangles: NFTs

Trans-endothelial electrical resistance: TEER

Thiobarbituric acid reactive substances: TBARS Dihydroxy-ethidium: DHE

Metalloprotease 9: MMP9

Zonula occuldin-1: ZO-1

6. Plan of Work

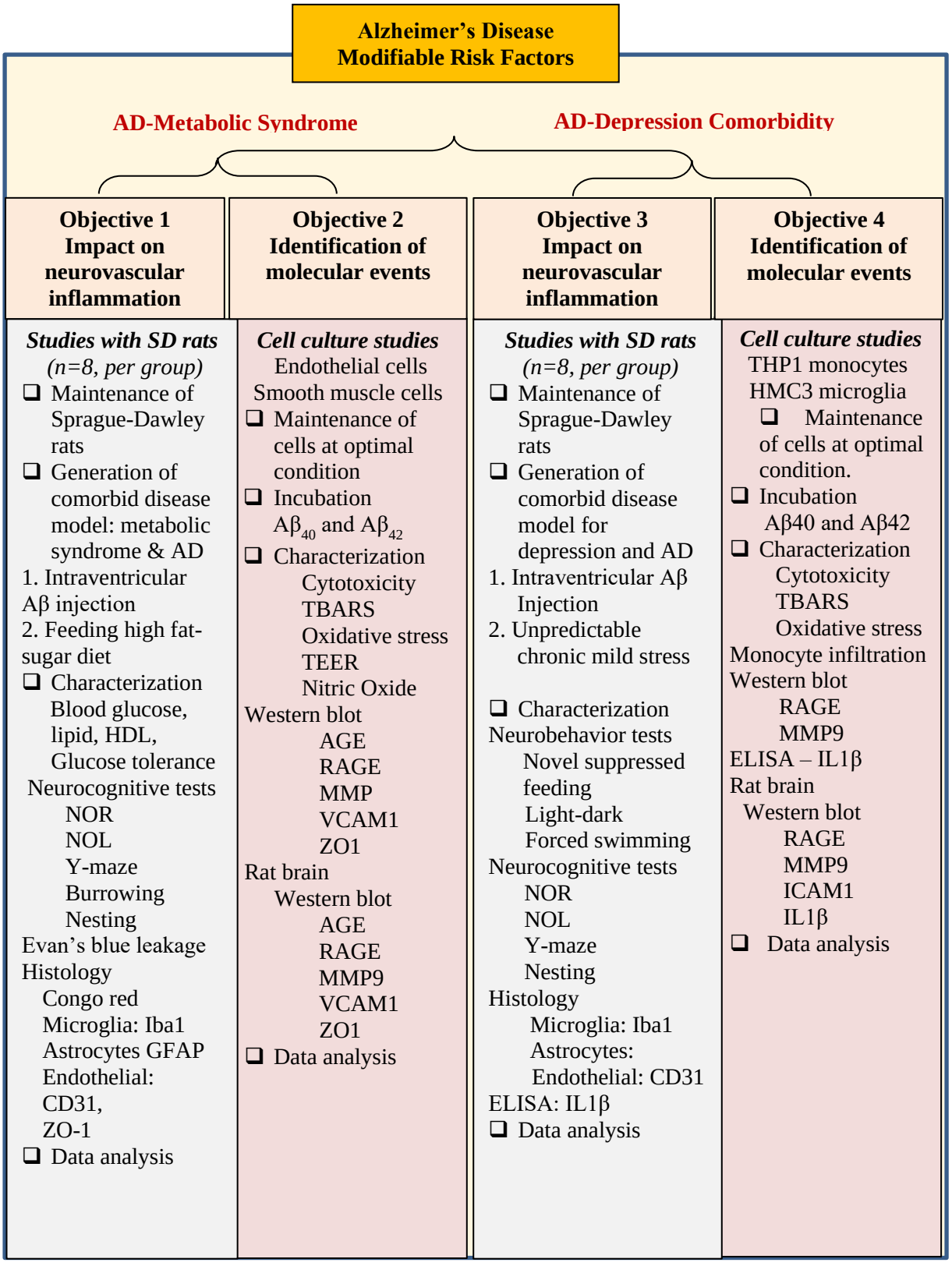

Figure 3. The details of the plan of work to accomplish the objectives.

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